

APPENDIX C:
“Clean” Version of Claim Listing

1. (Twice Amended) A fusion protein comprising a ubiquitin insert protein having an insert regulatory domain lying between an amino terminal and a carboxyl terminal of the ubiquitin insert protein, the insert regulatory domain being associated with a first quantity of free energy; and, a barnase target protein having a surface loop that begins at an alpha carbon of an initial amino acid of the surface loop and terminates at an alpha carbon of a terminal amino acid of the surface loop, the surface loop comprising a cytotoxic target domain of the barnase target protein, the cytotoxic target domain being associated with a second quantity of free energy, wherein, the ubiquitin insert protein is inserted at a point within the surface loop between the alpha carbon of the initial amino acid of the surface loop and the alpha carbon of the terminal amino acid of the surface loop, such that an amino-carboxyl length of the ubiquitin insert protein is at least two-times greater than an alpha-carbon-alpha-carbon length of the barnase target protein.

2 (Presently Amended) The fusion protein of claim 1, wherein the insert regulatory domain exists in either a folded or unfolded conformation and the target cytotoxic domain exists in either a folded or unfolded conformation, the insert regulatory domain and the target cytotoxic domain comprising a cooperative and reversible conformational equilibrium such that if the insert regulatory domain is in its folded conformation, the target cytotoxic domain is in its unfolded conformation and vice versa.

3. (Presently Amended) The fusion protein of claim 2, wherein all or part of the first quantity of free energy is made available to drive a folding of the target cytotoxic domain from its unfolded conformation by means of a first controllable effector signal, and all or part of the second quantity of free energy is made available to drive a folding of the insert regulatory domain from its unfolded conformation by means of a second controllable effector signal.

4. (Presently Amended) The fusion protein of claim 3, wherein the first controllable effector signal is selected from the group comprising ligand binding, pH, temperature, chemical denaturants, or mutations in either said insert domain or said target domain.
5. (Presently Amended) The fusion protein of claim 3, wherein the second controllable effector signal is selected from the group comprising ligand binding, pH, temperature, chemical denaturants, or mutations in either the insert domain or the target domain.
6. The fusion protein of claim 2, wherein the insert domain and the target domain are disabled from simultaneously co-existing in their respective folded conformations.
7. (Canceled) The fusion protein of claim 2, wherein said insert domain and said target domain are disabled from simultaneously co-existing in their respective unfolded conformations.
8. (Presently Amended) The fusion protein of claim 2, wherein any excess of the first quantity of free energy of the insert regulatory domain that is not necessary to stabilize the insert regulatory domain in its folded conformation is spontaneously transferred, through the structure of the fusion protein, to the target cytotoxic domain to unfold it from its folded conformation.
9. (Presently Amended) The fusion protein of claim 2, wherein any excess of the second quantity of free energy of the target cytotoxic domain that is not necessary to stabilize the target cytotoxic domain in its folded conformation is spontaneously transferred, through the structure of the fusion protein, to the insert regulatory domain to unfold it from its folded conformation.
10. (Presently Amended) The fusion protein of claim 2, wherein the insert protein comprises human ubiquitin, the insert regulatory domain comprises a regulatory domain of human ubiquitin, the target protein comprises barnase, the target cytotoxic domain comprises a cytotoxic domain of barnase, the amino-carboxyl length is about 38 Å, the initial amino acid of the surface loop comprises proline in the number 64 position ("Pro64"), the terminal amino acid

of the surface loop comprises threonine in the number 70 position ("Thr70"), and the alpha-carbon-alpha-carbon length is about 10.4 Å.

11. (Presently Amended) The fusion protein of claim 10 wherein the regulatory domain of human ubiquitin and the cytotoxic domain of barnase comprise a cooperative and reversible conformational equilibrium, that may be determined by the controllable first and second effector signals.

12. (Canceled) A method for the production of a protein comprising the steps of:

- a. selecting a linker containing first and second restriction sites between a Lys66 and a Ser67 codon of a barnase gene;
- b. using said first and second restriction sites of said linker to operationally insert a ubiquitin gene between two amino-acid codons of said linker, thereby creating a ubiquitin-barnase fusion gene;
- c. fully sequencing said ubiquitin-barnase fusion gene to verify its integrity;
- d. using enzymes to operationally insert said ubiquitin-barnase fusion gene into any plasmid of a BL21 (DE3) family, thereby creating an interim ubiquitin-barnase fusion expression plasmid;
- e. operationally inserting a gene for barstar and its natural promoter from *Bacillus amyloliquifaciens* into said interim ubiquitin-barnase fusion expression plasmid, thereby creating a ubiquitin-barnase fusion-barstar complex plasmid;
- f. cloning said gene for barstar into a T7 promoter-containing plasmid conferring resistance to an antibiotic other than ampicillin onto cells transformed by said T7 promoter-containing plasmid, thereby creating a barstar plasmid;

g. transforming *E. coli* BL21 (DE3) cells grown at about 20 to 37 degrees C in any medium compatible with *E. coli* growth using both said barstar plasmid and said ubiquitin-barnase fusion-barstar complex plasmid, and inducing said *E. coli* BL21 (DE3) cells with about 100 mg/L isopropyl b-D-thiogalactopyranoside;

h. harvesting said transformed *E. coli* cells by centrifugation after about 2 to 12 hours; after said induction;

i. placing said harvested *E. coli* cells in 10 mM sodium phosphate at a pH of 7.5, thereby creating a solution of harvested *E. coli* cells;

j. lysing said solution of harvested *E. coli* cells by repeated freeze-thaw cycles in the presence of about 10mg/liter lysozyme, thereby creating a lysate;

k. adding about 10 mg/liter DNase I to reduce the viscosity of said lysate;

l. centrifuging said reduced viscosity lysate to remove insolubles, thereby forming a supernatant;

m. adding about 8 M urea to said supernatant to dissociate bound barstar;

n. Removing said dissociated barstar from said supernatant by passing said supernatant through an anion exchange chromatography resin to yield a solution;

o. loading said solution onto a cation exchange column;

p. washing said solution with about 10 mM sodium phosphate (pH about 7.5) and about 6 M urea;

q. eluting said solution using a 0 to 0.2 M NaCl gradient;

r. Removing said urea from said dilution by dialysis against double-distilled water to yield barnase-ubiquitin fusion protein.